Mitigation of Hookworm Disease by Immunization with Soluble Extracts of *Ancylostoma ceylanicum*

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Hookworms are a leading cause of anemia in developing countries, and a strategy aimed at reducing pathology caused by blood-feeding adult parasites would be a valuable addition to global control efforts. This article describes experiments designed to induce resistance to the major clinical sequelae (weight loss and anemia) of *Ancylostoma ceylanicum* hookworm infection in Syrian golden hamsters of the outbred LVG strain. Previously infected animals acquired long-lived resistance to weight loss and anemia caused by a secondary hookworm infection. Furthermore, transfer of pooled serum from twice-infected hamsters to animals undergoing a primary infection was associated with partial resistance to growth delay and anemia. Active vaccination of hamsters with soluble adult hookworm antigens emulsified in alum led to partial protection from hookworm-associated pathology in the absence of reductions in adult worm burden. This intriguing result may have important implications for human vaccine development.

Blood-feeding hookworms, intestinal nematodes that currently infect more than a billion people worldwide, are a leading cause of anemia in developing countries [1, 2]. Hookworm infection takes a particularly devastating toll on the most vulnerable of the world’s population, including children and women of childbearing age [3–5]. It has been demonstrated that hookworm infection in children is associated with poor physical growth parameters and substandard school attendance and performance [6–11]. Pregnant women with hookworm are at particular risk for severe anemia, with potentially devastating consequences to both mother and fetus [5, 6]. Although effective anthelminthic agents for hookworm have been available for many years, reinfection typically occurs quickly after treatment [12, 13], and drug resistance has recently been documented in many years, reinfection typically occurs quickly after treatment [12, 13], and drug resistance has recently been documented in human isolates [14, 15]. In light of these issues, there is considerable interest in developing a vaccine against hookworm, as a means of improving the health of people living in highly endemic areas [16].

An animal model that reproduces the major clinical sequelae of the type of hookworm infection seen in humans would theoretically provide the best means of characterizing the major pathologic features of hookworm disease. This model also would have considerable utility for evaluating the effects of immunization against hookworm infection and pathology. These criteria are well satisfied by the Syrian golden hamster. When infected with *Ancylostoma ceylanicum* hookworms, golden hamsters exhibit the major clinical features observed in humans—namely, delayed growth and anemia [17, 18]. Immunization of hamsters with live *A. ceylanicum* larvae has been shown to protect against subsequent challenge infection when the larvae are administered (1) in a single dose [19, 20], (2) repeatedly in small “trickle” doses [21], or (3) as a single UV-irradiated dose [22]. It also has been demonstrated that immunization with soluble extracts or secretory products from adult *A. ceylanicum* worms leads to reduced worm burden in hamsters after subsequent challenge [23]. These findings, coupled with the inherent advantages of rodent models over larger species, make the hamster model of *A. ceylanicum* infection an attractive alternative to the canine model of *A. ceylanicum* [24] or *Ancylostoma caninum* [25] infection, for studies on hookworm pathogenesis and vaccine development. The fact that *A. ceylanicum*, unlike *A. caninum*, is a hookworm capable of completing its life cycle in humans [26–28] adds to the relevance of this model for work on the development of a human vaccine.

Here we present data from studies designed to induce resistance to the major clinical sequelae of hookworm infection (weight loss and anemia) in hamsters of the outbred LVG strain. Following an initial study to characterize the pathologic effects and humoral immune responses in this hamster strain during
primary *A. ceylanicum* infection, we investigated the level of resistance to disease on secondary infection. Studies were subsequently conducted using passive transfer of immune serum, as well as active vaccination with nonliving hookworm extracts (HEXs), to recapitulate the resistance to disease conferred by natural infection. Data from these experiments suggest that a vaccination strategy aimed at reducing hookworm-associated pathology by targeting adult parasites may be feasible.

**Methods**

**Parasites and hosts.** Infective third-stage larvae (L$_3$) of *A. ceylanicum* [29, 30] were generously provided by John Hawdon and Peter Hotez (Yale University School of Medicine, New Haven, CT), and the parasite life cycle was maintained as described elsewhere [17]. For adult worm recovery, we infected 3–4-week-old (40–60 g) male Syrian hamsters of the Lak:LVG(SYR)/BR outbred strain (Charles River Laboratories) with 150–200 L$_3$ by oral gavage. When adult worms developed (at least 21 days after infection), the animals were killed, and the parasites were harvested manually from the intestinal mucosa. Hookworms were rinsed with PBS and stored at −80°C until use. For studies of hookworm-associated pathology, hamsters were infected, as described above, with 50 L$_3$, and were followed for the times indicated in the figures. The approximate ages of the animals at the time of study are given in the figure legends. Because of the variable weight gain typical of this hamster strain, weight data are presented as percentages of values at day 0 (relative to the time of infection).

**Soluble HEX.** Previously frozen adult hookworms recovered 21–28 days after infection were manually homogenized in 50 mM Tris-HCl, pH 7.5, using a glass homogenizer [31]. Homogenates were sonicated briefly on ice (3 rounds of 5 half-second pulses, each separation by 30 s) and then centrifuged for 30 min at 12,000 g. Supernatants were removed, and the protein content of the soluble HEX was assayed by use of the BCA system (Pierce Chemical) and were assayed within 4 h of collection. Hemoglobin levels were measured using an assay kit (Total Hemoglobin kit; Sigma Diagnostics), following the manufacturer's protocol, with the following modifications. In brief, 10 μL whole blood was mixed into 2.5 mL Drabkin’s solution (prepared as directed, using reagents provided in the kit) in glass test tubes, and the samples were incubated for 15 min at room temperature. After incubation, sample tubes were vortexed, and 200 μL was transferred in duplicate wells to a microtiter plate. Samples were read at 530 nm in a microplate reader (MRX, Dynex Technologies) using a hemoglobin standard curve prepared from reagents provided in the kit.

**Analysis of antibody responses by ELISA.** HEX-specific antibody titers were measured as follows: microtiter plates (3915; Falcon) were incubated overnight at 4°C with 100 μL/well HEX at 5 μg/mL in Dulbecco’s PBS. The next day, plates were incubated for 1 h at 37°C, decanted, and rinsed 3 times with PBS containing 0.05% Tween 20 (PBS-T). Plates were blocked for 1 h at room temperature with 1% nonfat dry milk in PBS. Plates were rinsed 4 times with PBS-T after they were blocked and between each subsequent step. Hamster serum samples in duplicate were serially diluted in PBS-T to a final volume of 100 μL per well and were incubated for 1 h at 37°C. HEX-specific antibodies were then detected, using 100 μL/well horseradish peroxidase-labeled goat anti–hamster IgG (raised against heavy and light chain; ICN Biochemicals), were diluted 1:1000 in PBS-T, and were incubated for 30 min at 37°C. Bound secondary antibody was detected by the addition of 100 μL ABTS substrate solution (0.1% ABTS [Sigma] in 0.1 M citrate buffer, pH 5.0, and 0.03% H$_2$O$_2$) to each well. After a 30-min incubation at room temperature, A$_{405}$ was recorded using a microplate reader and antigen-specific titers were calculated by interpolating the dilution, giving an A$_{405}$ of 0.2 after subtraction of background. All values were normalized to a positive standard (serum from hamsters infected 102 days previously), which was included in each assay to control for day-to-day variation.

**Statistical analysis.** Data are presented as mean ± SE. Significance testing was conducted using statistical analysis software (StatView 4.51; Abacus Concepts). Analysis of variance was performed using Fisher’s protected least significant difference for multiple group comparisons, with *P* < .05 considered to be significant.

**Results**

**Hookworm infection causes delayed weight gain in outbred hamsters.** Male weanling outbred LVG hamsters were infected with 50 *A. ceylanicum* L$_3$, by oral gavage on day 0, and their weights were compared with those of uninfected controls for 105 days. Uninfected controls (initial mean weight, 71.4 ± 0.8 g) gained weight rapidly throughout the observation period, approaching a mean of 150% of their initial mean weight by day 14 and 200% by day 56 and exceeding 225% (162.0 ± 8.9 g) by day 105 (figure 1A). Hookworm-infected hamsters (initial mean weight, 72.2 ± 1.3 g) also exhibited rapid growth early in the observation period. However, by 8 days after infection, they began to exhibit significant growth delay, and they did not have a net increase in weight from day 14 to day 36. By contrast, uninfected animals gained ~35% of their initial weight (~25 g) in this interval. Furthermore, despite the fact that infected hamsters resumed gaining weight after day...
Weight (A), blood hemoglobin levels (B), and hookworm-specific antibody responses (C) in hamsters after a primary infection with *Ancylostoma ceylanicum*. Hamsters (∼30 days of age) were infected on day 0 with 50 infective third-stage A. ceylanicum larvae (50 L3; n = 5) or were left uninfected (0 L3; n = 6). Normalized weights (A) were determined by calculating the percentage of the value at day 0 for each animal. All values are mean ± SE. HEX, hookworm extract.

36, with kinetics that resembled those of the uninfected animals, the groups remained separated by at least 30% of their initial weight (i.e., >20 g) 36–105 days after infection (P ≤ .03 at all time points measured from day 8 to day 105).

**Hookworm infection causes anemia in outbred hamsters.** Blood hemoglobin levels in uninfected control hamsters increased from a mean of 15.4 g/dL at day 7 to 18 g/dL by day 28, remaining between 18 and 20 g/dL for the remainder of the observation period (figure 1B). In contrast, hemoglobin levels in the infected group decreased from a mean of 15.2 g/dL at 7 days after infection to a nadir of 12.8 g/dL by day 36 (representing a 34% reduction vs. the uninfected controls at that time point; P = .0003). Coincident with the observed resumption of weight gain (figure 1A), hemoglobin levels began to rise steadily after day 36 in the infected animals, reaching levels statistically equivalent to those of the uninfected hamsters by day 77.

**Specific immune response to a primary hookworm infection.** Figure 1C shows hookworm-specific humoral immune responses in infected hamsters, as measured by ELISA. Specific antibody titers were undetectable or present at low levels for the first 14 days after infection and then began to rise steadily, exceeding a mean of 500 by day 36 (the time point corresponding to the onset of improvements in weight gain and hemoglobin level; figure 1). Antibody titers continued to rise in the infected animals throughout the observation period, exceeding a mean of 2400 by day 105.

**Previously infected hamsters are resistant to severe hookworm disease on secondary challenge.** To determine whether hamsters that had recovered from the pathologic effects of a primary hookworm infection would be resistant to disease on secondary challenge, animals from the experiment depicted in figure 1 were infected with 50 L3 and observed as before for signs of hookworm-associated pathology. As shown in figure 2A, after chal-
lengue infection, previously naive hamsters (i.e., the 0/50 [primary/secondary infection] L3 challenge group with an initial mean weight of 164.1 ± 9.1 g) exhibited considerable weight loss, reaching a nadir of ∼90% of prechallenge weight by day 39. These animals subsequently demonstrated rapid weight gain, returning to slightly >100% of their mean prechallenge value by day 60. In contrast, previously infected hamsters challenged with a second infection (i.e., the 50/50 [primary/secondary infection] L3 challenge group with an initial mean weight of 139.4 ± 7.0 g) never dropped below 100% of their mean prechallenge weight in the period after challenge; instead, their weight gradually increased to >100% by day 60.

Blood hemoglobin levels in the 0/50 L3 hamsters remained relatively stable for the first 14 days after challenge infection and then declined from a mean of 19 g/dL to 14.3 g/dL by day 32 (a 25% decrease) (figure 2A). Hemoglobin levels remained depressed in these animals for the next 9 days, and, as observed previously (figure 1), this anemia was coincident with the period of weight loss (figure 2A). A recovery in blood hemoglobin levels began after day 41, reaching a mean of 19.6 g/dL by day 56. Hemoglobin levels in the 50/50 L3 group also declined after challenge infection, dropping to 16.5 g/dL by day 21. However, these animals experienced an accelerated recovery from anemia, with values rebounding to 20 g/dL by day 41. Adult worms were recovered from the animals at 61 days after challenge, but the yield was rather low, with a mean of 2.5 ± 0.8 parasites (range, 0–5 parasites) in the 0/50 L3 group and 0.7 ± 0.3 parasites (range, 0–1 parasite) in the 50/50 L3 group.

Reduction of hookworm disease on passive transfer of immune serum. To ascertain if the resistance to hookworm disease observed on secondary infection could be passively transferred, we prepared H-2x from the 50/50 L3 group 61 days after the second infection and used it in a passive immunization study. The hookworm-specific titer of H-2x was comparable to that in singly infected hamsters at day 105. Two groups of weanling hamsters (initial mean weights, 57.0 ± 1.0 g and 56.6 ± 1.5 g) were infected with 50 L3, and, at day 11, 0.5 mL of pooled H-2x or NHS was administered to each animal. The serum transfer was timed to immediately precede the onset of weight loss and anemia subsequent to hookworm infection. Also, hookworms reach the blood-feeding stage at approximately this time point. Weight gain and blood hemoglobin levels in the treated animals were compared with those in an uninfected control group (initial mean weight, 52.5 ± 1.4 g). Uninfected control hamsters exhibited rapid weight gain throughout the experiment, whereas infected hamsters treated with NHS began to exhibit delayed weight gain by day 14 (figure 3A). By day 46, the normalized weight of the NHS group was lower than that of the uninfected controls by ∼40% of the prechallenge value (corresponding to an actual difference of >12 g), although the difference was not statistically significant (P = .11). In contrast to results obtained from the NHS group, a difference between the H-2x control group and the uninfected group was not detected until day 25, and the disparity remained relatively small (∼15% of prechallenge weight; P > .49) for the remainder of the experiment.

Blood hemoglobin levels in the uninfected control group increased gradually from a mean of 19.6 g/dL at day 14 to 21.9 g/dL at day 46. In the NHS group, values declined from 19 g/dL at day 14 to 17.1 g/dL at day 21, representing an 18% reduction versus that in the uninfected group at day 21 (P < .0001). After 21 days, values increased somewhat in the NHS group but remained significantly below those of the uninfected controls for the remainder of the experiment (P ≤ .003 at each time point). Hemoglobin levels also decreased in the H-2x group after infection, but, in contrast to the NHS group, this drop was not observed until after day 17 in the H-2x animals. Furthermore, an accelerated recovery of hemoglobin levels was observed in H-2x hamsters by day 32, and levels in the H-2x group were significantly higher than those in the NHS group on days 42 and 46 (P < .02 at each time point). Worm burden was determined on day 46, and no significant difference was observed between the H-2x group (1.6 ± 0.6 parasites) and the NHS group (2.4 ± 0.9 parasites).

Vaccination with adult HEX confers partial protection against...
To determine whether active immunization could protect animals from hookworm infection and/or disease, we immunized weanling hamsters with 200 µg adult HEX in alum and administered 2 booster immunizations of 100 µg HEX in alum at 14-day intervals. One week after the second booster immunization, the animals were challenged with 50 L₃. As shown in figure 4A, repeated immunization with HEX led to steadily increasing specific IgG titers that were comparable in magnitude to those induced by infection (figure 1C). After challenge infection, anti-HEX titers increased gradually over the first 28 days, from a mean of ~800 at day 0 to >1000 at day 28, and then increased more substantially to reach a mean of ~1500 by day 34. Anti-HEX responses were below detectable limits in alum control animals until day 21, after which time they began to increase as a consequence of the challenge infection. Titers in the alum controls did not approach those of the vaccinated hamsters during the experiment. As expected, anti-HEX responses were below detectable limits in uninfected animals at all times measured.

Figure 4B shows the effects of HEX immunization on body weight after hookworm infection. As expected, uninfected control hamsters (mean weight, 119.3 ± 4.3 g at day 0) steadily increased in weight throughout the experiment, reaching a mean of >115% of their day-0 weight by day 34. Infected alum control animals (mean weight, 112.0 ± 5.3 g at day 0) began to lose weight after day 14, dropping from 105% to <95% of their prechallenge weight by day 34 (P ≤ .008 for alum controls vs. uninfected animals at all time points after day 14). HEX-vaccinated hamsters (mean weight, 121.7 ± 5.8 g at day 0) did not continue to gain weight after day 14; however, they were refractory to hookworm-associated weight loss during days 18–34, the time period when alum controls were exhibiting profoundly declining weight (P ≤ .04 for HEX-vaccinated animals vs. alum controls at all time points during days 18–34).

Figure 4C shows the effects of HEX immunization on blood hemoglobin levels after challenge infection. While hemoglobin levels decreased in HEX-vaccinated hamsters on challenge, this decline occurred more slowly and to a lesser extent than that in infected alum control animals. Most notably, the HEX-vaccinated hamsters exhibited a rapid early recovery in hemoglobin levels, which were statistically equivalent to levels in uninfected controls and significantly higher than levels in alum controls by day 34 (P = .002 for animals vaccinated with HEX in alum vs. alum controls at day 34).

To determine whether HEX vaccination had any impact on adult worm burden, we killed hamsters at day 34 and recovered parasites. Despite the positive effects on hookworm-associated weight loss and anemia observed in HEX-vaccinated animals (figure 4B, 4C), there was no apparent effect on worm burden: The mean number of worms recovered from the HEX group was 7.5 ± 0.8 parasites, versus 7.3 ± 1.8 parasites in the alum control group.

**Discussion**

Herein we have described experiments designed to induce resistance to the major clinical sequelae of hookworm infection. Using a model of *A. ceylanicum* infection in the outbred LVG
strain of Syrian golden hamster, we have demonstrated that previously infected animals acquire long-lived resistance to weight loss and anemia caused by a secondary hookworm infection. Furthermore, transfer of pooled serum from the twice-infected hamsters to animals undergoing a primary infection was associated with partial protection from growth delay and anemia. To our knowledge, this is the first report of passive transfer of protection from disease in the hamster model of *A. ceylanicum* infection. We also demonstrate for the first time that active vaccination of hamsters with a nonliving vaccine consisting of soluble hookworm antigens leads to partial protection from hookworm-associated pathology in the apparent absence of an effect on adult worm burden.

Children infected with hookworm exhibit anemia and delayed growth (reviewed in [4]). In their landmark study of >1800 children in the rural southern United States, Smillie and Augustine [7] found that teenagers with moderate or heavy *Necator americanus* hookworm infections were, on average, 4.5 and 6.8 kg underweight, respectively, compared with uninfected controls from the same area. Furthermore, heavily infected children also had 9%–21% reductions in hemoglobin level, compared with uninfected controls. Similarly, we found that young male hamsters of the outbred LVG strain with sublethal *A. ceylanicum* infection became anemic and exhibited growth delay that persisted despite eventual resolution of the anemia (figure 1). Menon and Bhopale [18] also found that young male hamsters (strain unspecified) became anemic within 13 days of *A. ceylanicum* infection, although a statistically significant effect on weight gain was not detected in that study until day 30.

As in young animals, we observed that previously naive adult hamsters quickly became anemic after infection and ultimately rebounded to normal hemoglobin levels (figure 2B). We found that the effect of *A. ceylanicum* infection on weight was also transient in these older animals, with a return to 100% of prechallenge weight occurring by 60 days after infection (figure 2A). Thus, our data indicate that certain pathologic effects of primary *A. ceylanicum* infection may be variable, depending on the age of the hamster at the time of infection. Furthermore, the strain of hamster also may affect the pathologic outcome. To illustrate, in a study that used inbred male DSN hamsters infected at 8–12 weeks of age, Garside and Behnke [17] reported, in contrast to our observations using the outbred LVG strain (figure 2), that adult animals manifested chronic anemia for up to 100 days and reduced weight for >60 days.

Analysis of hookworm-specific antibodies by ELISA demonstrated that hamsters undergoing primary *A. ceylanicum* infection acquired vigorous serum IgG responses that continued to increase throughout the 105-day observation period (figure 1C). These results are in general agreement with those of Garside et al. [32], who used DSN hamsters infected with a comparable dose of L3 and a 70–80-day observation period [32]. Persistent hookworm-specific antibody responses also have been detected in dogs [24] and in humans [33] infected with *A. ceylanicum*. In contrast, Menon and Bhopale [18] and Kamath et al. [34] found that hookworm-specific serum antibody titers (as measured by indirect hemagglutination) declined in hamsters after peaking at day 60 and days 45–55, respectively. The relevance of serum IgG responses has not yet been established in this model; however, given the blood-feeding activities of the adult worms, it is conceivable that IgG could neutralize molecules involved in pathogenesis (which are presumably secreted at the site of attachment) or attack the worms directly (see below).

In contrast to serum antibody responses, relatively little is known concerning mucosal antibody responses in this model, although hookworm-specific antibodies have been detected by immunoblot in gut washings of twice-infected hamsters [19]. The isotype of the gut-associated antibodies was not determined in that study [19]; however, the IgA class has been shown to be enriched in hamster intestinal secretions [35, 36]. Hookworm-specific secretory IgA might function by neutralizing parasite secretory molecules. Further studies will be necessary in order to characterize the mucosal antibody response in this model and to determine its pertinence for protective immunity.

After primary exposure to *A. ceylanicum*, hamsters can mount protective immune responses that reduce worm burden on secondary challenge [19–21]. The basis for the protective immunity in this model has not been definitively established. However, in addition to elevations in hookworm-specific antibody, immune animals exhibit an accelerated mucosal mast cell response [19, 20]. Studies of protective immunity in the hamster model typically have been conducted using inbred animals and relatively short intervals between priming and challenge infections. Our results complement and extend these findings by demonstrating that resistance to weight loss and severe anemia after secondary challenge also may be induced in outbred LVG hamsters, and, furthermore, this resistance persists for at least 105 days after the primary exposure (figure 2). Preliminary data suggest that adult worm burden also may be reduced on secondary infection, and further work is planned to examine worm survival at various times after challenge in this model.

Although passive transfer of immune serum has been shown to reduce lung L1 burden in mice infected with *A. caninum* [37], the nonpermissive nature of the murine model does not allow for the study of pathology caused by adult hookworms. We found that transfer of pooled serum from twice-infected hamsters to animals undergoing a primary infection was associated with improved growth and hemoglobin status (figure 3). To our knowledge, this is the first report of passive transfer of resistance to disease in this permissive model of hookworm infection. The protective factor in H-2x is likely to be hookworm-specific antibody; however, the possible contributions of other serum constituents that may be elevated in H-2x, such as activated complement and/or inflammatory cytokines, cannot be discounted at this time. Since serum transfer was timed to co-
incide with the onset of blood feeding by the parasites (and thus could not have affected any previous developmental stage), we hypothesize that antibodies in the H-2x serum acted by neutralizing certain molecules secreted by the adult worm to facilitate feeding and survival in the host gut, such as anticoagulants [31, 38, 39], antiplatelet molecules [40], and protease inhibitors [41]. Alternatively, antibodies in H-2x may have acted directly on the adult worms, causing, for example, accelerated attrition via complement activation and cell-mediated cytotoxicity. Additional passive transfer experiments will be necessary to characterize the molecular targets of H-2x and to confirm the identity of the protective factor(s).

We show here that hamsters vaccinated with soluble A. ceylanicum extract emulsified in alum (the only adjuvant currently approved for use in humans) were partially protected from disease (figure 4). Vaccination with UV-attenuated L, has been shown to induce protective immunity to A. ceylanicum infection in hamsters [22]; however, aside from the study presented in this paper, we are aware of only one other published report of a nonliving vaccine being used in this model. In that study, Khan et al. [23] employed soluble adult worm extracts or excretory-secretory antigens administered to male hamsters (strain unspecified) with Freund’s complete adjuvant. Depending on the antigen and dose, 25%-67% reductions in adult worm burden were observed at day 21, although no pathologic data were presented. The authors also found that the level of parasite-specific serum IgG responses was correlated with the degree of protection, although a definitive role for IgG was not established. We also observed robust IgG responses in HEX-vaccinated animals (figure 4A); however, in contrast to the results of Khan et al. [23], we found no apparent reduction in worm burden, despite the clinical improvements observed in the vaccinated hamsters (figure 4). The basis for this disparity is unknown; however, differences between the two studies in time of worm recovery, hamster strain, immunization protocol, and/or adjuvant may provide some explanation. Additional studies will be necessary to determine whether the kinetics of worm attrition are affected in HEX-vaccinated animals, since we cannot rule out the possibility that a more rapid initial reduction of adult worm burden might have had a positive impact on disease.

In summary, the data presented here provide preliminary evidence that vaccination protocols designed to prevent disease caused by adult hookworms may be feasible. This intriguing result may have important implications for vaccine development. Indeed, because the induction of sterile immunity by antihelminth vaccines may prove to be a difficult goal [42], an approach that specifically targets adult worm antigens involved in pathogenesis might be an attractive strategy. Additional vaccine studies are ongoing to further evaluate this approach, using HEX in additional doses and schedules, excretory-secretory products, and defined recombinant molecules.

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